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Plant growth and development influenced by transgenic insertion of bacterial chitinolytic enzymes

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Abstract

The role of the chitinolytic enzymes in plants is not necessarily restricted to plant defense. Tomato plants transformed with an endochitinase and a chitobiosidase gene from *Streptomyces albidoflavus* and growth under greenhouse conditions showed a significant reduction in plant height, and reduced time to flowering compared with the control (non-transformed) plants. The levels of chitobiosidase and endochitinase activity in the transgenic tomato plants were positively correlated with early flowering, and negatively correlated with plant height. We have not determined whether these effects are exclusively due to the expression of the transgenes of endochitinase and chitobiosidase from *S. albidoflavus* or the additive effect of these 2 enzymes combined with the endogenous chitinolytic enzymes produced by the plants. However, when control plants were trimmed, early flowering was observed compared with the controls that were not trimmed, which indicates that wound induced proteins such as chitinolytic enzymes affect the time of flowering. In addition, the expression of the endochitinase and chitobiosidase genes significantly increased the number of flowers and fruit on the plants, resulting in an increase in yield of fruit. One of the primary goals of crop breeding programs is to increase the productivity of plants. These two genes were directly associated with plant productivity, and should be studied further.

Introduction

Plant chitinolytic enzymes and β-1–3 glucanases are among a group of proteins that are inducible in plants in response to various forms of stress, and are generally believed to serve protective functions in the plants, although the exact nature of those functions is not clear (Boller 1987). Chitinolytic enzymes have a role as phytochemical defense agents against pathogenic fungi as indicated by (1) the co-ordinated induction of those enzymes in response to pathogen invasion (Roby and Esquerre-Tugaye 1987), (2) the fact that chitinolytic enzymes from plants are potent inhibitors of fungal spore germination and mycelial growth *in vitro* (as demonstrated by their ability to hydrolyse fungal cell walls) (Broekaert et al. 1988; Mauch et al. 1988; Roberts and Selitrennikoff 1988; Schlumbaum et al. 1986), (3) higher levels of chitinolytic activity in resistant cultivars compared with susceptible cultivars (Hughes and Dickerson 1991; Vogelsang and Barz 1990), (4) enhanced resistance of plants following transformation with chitinolytic enzymes (Broglie et al. 1991; Nishizawa et al. 1999; Tabei et al. 1998), and (5) enhanced resistance following constitutive expression of chitinolytic enzymes (Lin et al. 1995). However, not all of the isoforms of chitinolytic enzymes produced in plants have antifungal properties. Schickler and Chet (1997) indicated that the phenomenon of variable antifungal potency among chitinolytic enzymes is problematic. The success of the defense mechanism depends on both the type of chitinolytic enzyme and the species of fungus. Not all plants that have been transgenically enhanced for chitinolytic activity, and that express high levels of chitinolytic enzymes, exhibit the expected increase in resistance to fungal pathogens

(Schickler and Chet 1997). Sela-Buurlage et al. (1993) showed that only class I vacuolar chitinolytic enzymes and ß-1–3-glucanase isoforms from tobacco exhibited antifungal activity against *Fusarium solani*, while the class II isoform of both enzymes exhibited no antifungal activity (Sela-Buurlage et al. 1993).

The possibility that chitinolytic enzymes are involved in non-defensive roles is beginning to be elucidated (Patil and Widholm 1997). So far, chitinolytic enzymes have been shown to be involved in many plant process such as flowering (Neale et al. 1990), reproduction (Leung 1992), germination (Vogeli-Lange et al. 1994; Wu et al. 1994), somatic embryogenesis (De Jong et al. 1992; Dong and Dunstan 1997; van Hengel et al. 1998), plant growth (Patil and Widholm 1997; Spaink et al. 1993), fruit ripening (Robinson et al. 1997), and senescence (Hanfrey et al. 1996).

One issue that has not been completely addressed is the identification of the target substrate for the chitinolytic enzymes in plant. Until recently it was believed that no substrates for chitinolytic enzymes were present in plants. Flach et al. (1992) stated that the interest in plant chitinolytic enzymes is partly due to the probable absence of natural substrates in the plant itself (Flach et al. 1992). However, chitinolytic enzymes catalyze the hydrolysis of chitin, a linear homopolymer of ß 1–4-linked N-acetylglucosamine (GlcNAc) residues and immunological studies have revealed the presence of GlcNAc residues in the secondary cell wall of plants, probably in the form of glycolipids (Benhamou and Asselin 1989; Benhamou et al. 1990). In addition, proteins associated with the tobacco nuclear pore complex that have oligosaccharides attached to the terminal N-acetyl glucosamine residues have been identified (Benhamou and Asselin 1989; Heese-Peck et al. 1995). These results have led to the belief that chitinolytic enzymes may have a non-defensive function in plants, such as digestion of the plant cell wall material (Vogeli-Lange et al. 1994), cell division, differentiation, and development (Collinge et al. 1993; Patil and Widholm 1997).

In the current study we transformed tomato (*Lycopersicon esculentum*) cv. Beefmaster with an endochitinase and a chitobiosidase gene from *Streptomyces albidoflavus*. The T1 and T2 transgenic tomato plants (BmB1) that expressed higher levels of chitinolytic activity than the non-transgenic plants had enhanced resistance against herbivorous insects, as indicated by the reduced growth and development of the cabbage looper *Trichoplusia ni* (Huber) following ingestion of the foliage (Gongora et al. 2001). In addition, these transgenic plants showed a significant reduction in height, and displayed earlier flowering than the control plants. To determine if this effect was due to the expression of the transgenes in the plants or to a mutational effect resulting from the insertion of the transgenes in the plant genome of the BmB1 line, seeds from ten different T1 transgenic lines were planted, and monitored for plant height, number of leaves, time from transplanting to flowering, and expression of chitinolytic enzymes. In addition, the effect of trimming leaves from plants was evaluated. Numerous literature reports have shown that manual removal of lateral shoots activates a "wound-response" or an "induced response" in the plant (Karban and Myers 1989), and numerous authors have demonstrated that chitinolytic enzymes are wound inducible proteins. These experiments help us understand the role of chitinolytic enzymes in plant development.

Materials & methods

Gene cloning and plant transformation

The endochitinase and chitobiosidase genes isolated from a genomic DNA library from *Streptomyces albidoflavus* were engineered in the binary vector pBin 19 (Gongora et al. 2001). The plasmid product, identified as p*S. a*-endochitinase-chitobiosidase (Figure 1), was used for transformation of the tomato cultivar Beefmaster VFN (BM). The transformation and regeneration was accomplished by a modification of McCormick et al. (1986) reported by Xue et al. (1994). *Agrobacterium tumefaciens* LBA 4404 containing the construct p*S.a*-endochitinase-chitobiosidase was used. The selection of the transformed tissue was done on a selection medium containing 50 mg l^{-1} of kanamycin and 500 mg l^{-1} of carbenicillin. Following transformation and regeneration, the tomato plants were transferred to soil, mantained in the greenhouse and allowed to grow until they produced fruit. The seeds were collected for further use.

PCR verification for transformants

DNA was isolated from the leaves of the tomato transgenic and control plants growing in the greenhouse, using the minipreparation method described by Cheung et al. (1993). The presence of the *nptII* marker gene was determined by PCR amplification.

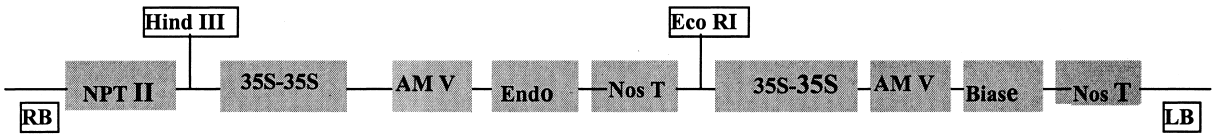

Figure 1. p*S.a*-endochitinase-chitobiosidase: Schematic diagram of the T-DNA endochitinase-chitobiosidase transformation plasmid. The plasmid contains the NPTII, neomycin phosphotransferase gene as selective gene, the double cauliflower mosaic virus 35S-35S constitutive promote (CaMV35S(Ca)), the translator enhancer of the alfalfa mosaic virus (AMV), the endochitinase and the chitobiosidase genes from *Streptomyces albidoflavus* and the Nos terminador sequence.

The primers NPT II-F (5' CCCCTCGGTATCCAATTAGAG 3') and NPTII-R (5' CGGGGGGTGGGCGAAGAACTCCAG 3') were chosen to amplify the region of the T-DNA containing the *nptII* gene. Standard PCR reaction was preformed including a cycle of 94 °C × 5 min 1 cycle, 94 °C × 30 s, 67 °C × 30 s, 72 °C × 1 min 30 cycles, 72 °C × 5 min 1 cycle. A band of 508 bp indicated the presence of the gene (data not shown). Transgenic plants that were *nptII* negative were discarded.

T2 Tomato lines BMB1-1/7: relationship of endochitinase and chitobiosidase activity to plant size and flowering time

The levels of endochitinase and chitobiosidase expression in the plants were quantified by enzymatic activity assays using methyl umbelliferyl substrates. Leaf samples (10 to 15 mg) were homogenized in 150 μl of buffer (0.1% SDS, 0.1% Triton X-100, 10 mM Na2 EDTA, 10 mM 2-mercaptoethanol, 100 mM sodium acetate buffer, adjusted to pH 4.8) per mg of tissue with an electric motor-driven Kontes pestle for about 30 s, and stored on ice. Samples were centrifuged (16,000 × *g*, 4 °C, 5 min) and an aliquot (100 μl) of the supernatant solution was mixed with 40 μl of substrate (1 mg methyl umbelliferyl N,N,N triacetyl chitotriose or methyl umbelliferyl N,N,N triacetyl chitobiose in 3.5 ml of 100 mM sodium acetate buffer, pH 5.0). After incubation at 28 °C for 30, 60, 90, 120 or 150 min the reaction was stopped by mixing an aliquot (20 μl) of the reaction mixture with 180 μl of 0.2 M Na_2CO_3. Fluorescence was measured with a CytoFluorII scanner using the CytoFluorII program (Excitation: 360/40 nm; Emission: 460/40 nm; Gain: 70). A standard curve was calculated based on the fluorescence of 4-methylumbelliferone (0, 20, 40, 60, 80, 100, 150, 200 mM) dissolved in 0.2 M Na_2CO_3.

Protein concentration was determined for each sample using the Bio-Rad protein assay, based on the Bradford dye-binding procedure (Bio-Rad). Bovine serum albumin standards, dissolved in umbelliferyl assay buffer, were prepared at concentrations of 0, 20, 50, 80 and 120 μg. The reagent was prepared by diluting the Bio-Rad protein assay dye reagent 1:5 with deionized water. Aliquots (200 μl) of the diluted reagent were transferred to a 96 well microtiter plate, and 2 μl of standard or leaf extract was added. After mixing, the plates were read in a SLT Spectra ELISA plate reader at 620 nm. Activity slopes (nM MU /min) were determined for each sample, and the value of nM MU/min was expressed relative to the amount of protein present (nM MU /min/μg protein).

The height of the plants was determined 30 to 40 days post-seed planting and the flowering time was measured by counting the days from transplanting to the formations of the first flowering buds.

Identification of T1 transgenic lines

The presence of the *nptII* marker gene was determined by PCR amplification. Southern analyses were performed on the T1 plants to determine the copy number of the T-DNA, endochitinase gene and chitobiosidase gene. DNA was isolated from leaflets from the plants growing in the greenhouse using a modification of the miniprep technique described by Fulton et al. (1995). The probe for the chitobiosidase gene was designed using the chitobiosidase gene inserted in the plasmid pBluescript II SK^- (Stratagene, La Jolla, CA), The probe for the endochitinase gene was designed using the endochitinase gene excised from the p*S.a*-endochitinase plasmid (endochitinase gene inserted in pBin19) (Gongora et al. 2001).

Relationship between chitinolytic enzyme activity and plant development T1 tomato lines

After determining that the T1 lines contained the endochitinase and chitobiosidase transgenes, fully developed leaflets from the first leaves of the plants were evaluated for endochitinase and chitobiosidase activity at 30, and 45 days post-seed planting. The

height of each of the plant was measured 45 days post-seed planting, the number of leaves on each plant was counted, and the time from transplanting to flowering was noted.

Effect of leaf trimming on T2 transgenic tomato line

Between 10 and 20 control plants and T2 BmB1-1 were trimmed by manually breaking off the lateral leaves when they started to grow. Another set of control and transgenic plants, that were planted at the same time, was allowed to grow without leaf removal. The flowering time post-transplanting and the number of fruits produced on the plants 60 days post-transplanting were noted.

Results

Relationship between chitinolytic enzyme activity and development of T2 Tomato lines

Young, fully developed leaflets from the first leaf of control (non-transgenic) Beefmaster tomato plants and T2 transgenic tomato plants (BmB1-1/7), expressing the endochitinase and chitobiosidase genes from *S. albidoflavus*, were evaluated for endochitinase and chitobiosidase activity at 30 and 45 days post-seed planting. The determination of the activity was performed on these days because at 30 days the seedlings were ready to be transplanted to individual pots, and at 45 days the transgenic plants initiated the formation of the flowering buds.

Two different sets of plants were used for these analyses. In addition to the 30 and 45 day analyses of enzyme activity, chitinolytic activity was measured in set I at 105 days post-seed planting (after transgenic plants produced fruit), while the third measurement for set II was performed at 75 days post-seed planting (when transgenic plants were producing fruit).

For Set I, 20 control and 20 T2 BmB1-1 plants. ANOVA indicated that for all three time intervals (30, 45 and 105 days post-seed planting) endochitinase activity was significantly different when comparing control and transgenic plants [30 days $F = 28.78$, $p < 0.001$; at 45 days $F = 38.52$, $p < 0.001$; and 105 days $F = 4.95$, $p = 0.035$ (Figure 2A)]. For chitobiosidase activity, the difference was significant between the control and transgenic plants at 30 days ($F = 62.74$, $p < 0.001$) and 45 days ($F = 28.13$, $p < 0.0001$), but not at 105 days ($F = 0.34$, $p = 0.564$) (Figure 2B). In the

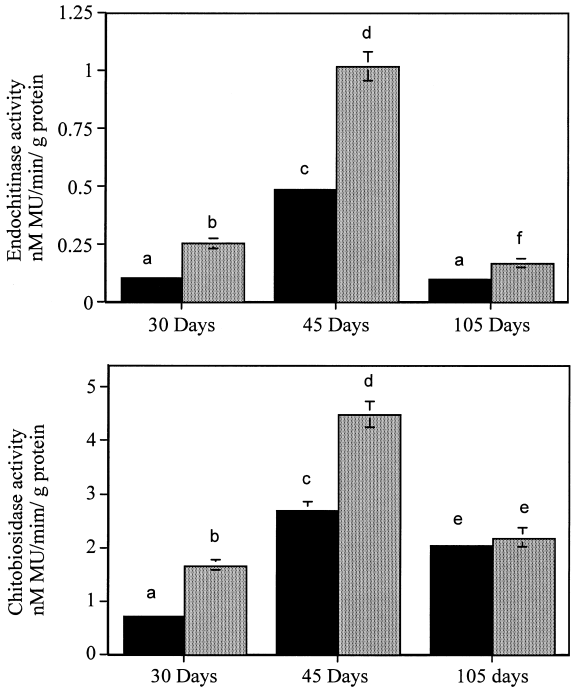

Figure 2. 2A–2B. Endochitinase and chitobiosidase activity respectively in tomato plants from Set I at 30 days (prior to transplant), 45 days (time of flowering of transgenic plants) and 105 days (senescent plants) post-planting. Black bars represent the mean endochitinase or chitobiosidase activity in control Beefmaster. Gray bars represent the mean endochitinase or chitobiosidase activity T2 transgenic Beefmaster B1 line. Vertical lines indicate ± 1 SE. Columns associated with the same letter are not significantly different.

transgenic plants, when the levels of activity of both endochitinase and chitobiosidase were compared over time, the difference was significant, (for endochitinase 30 vs. 45 days $F = 134$, $p < 0.001$; 45 vs. 105 days $F = 161$, $p < 0.001$). The control plants showed the same response, (for endochitinase activity 30 vs. 45 days $F = 130$, $p < 0.001$; 45 vs. 105 days $F = 88.28$, $p < 0.001$).

For Set II, 20 control and 30 BmB1-1. ANOVA indicated that, at the three points in time (30, 45 and 75 days post-seed planting) the difference was significant between control and transgenic plants for both endochitinase activity and chitobiosidase (Figure 3A and 3B): endochitinase activity, at 30 days $F = 18.4$, $p < 0.001$, at 45 days $F = 41.17$, $p < 0.001$ and at 75 days $F = 14.48$, $p = 0.035$); similar results were obtained for chitobiosidase activity. When the enzyme activity was compared at different times for the transgenic plants, there was a difference in the endochitinase activity when comparing 30 days and 45 days ($F = 73.65$, $p < 0.001$), but no difference occurred between

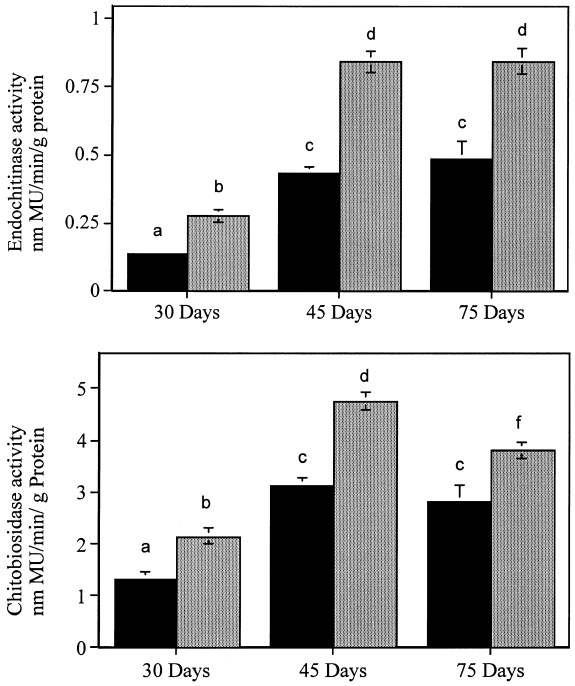

Figure 3. 3A–3B. Endochitinase and chitobiosidase activity respectively in tomato plants from Set II at 30 days (prior to transplant), 45 days (time of flowering of transgenic plants) and 75 days (flowering of control plants and fruiting of transgenic plants) post-planting. Black bars represent the mean endochitinase or chitobiosidase activity in control Beefmaster. Gray bars represent the mean endochitinase or chitobiosidase activity in T2 transgenic Beefmaster B1 line. Vertical lines indicate ± 1 SE. Columns associated with the same letter are not significantly different.

45 and 75 days. In the case of the chitobiosidase activity, there was a significant difference at all three times ($F = 71.94$, $p < 0.001$). For the control plants, the difference was significant between 30 and 45 days for both endochitinase activity and chitobiosidase activity. There was a difference between 30 and 45 days for endochitinase activity ($F = 57.05$, $p < 0.001$) and for chitobiosidase activity ($F = 38.50$, $p < 0.001$). However, no difference occurred between 45 and 75 days for either enzyme.

The height of the plants was determined 40 days post-seed planting (Figure 4). ANOVA indicated that there was a significant difference between the height of the control and transgenic plants (set I, $F = 159.0$, $p < 0.001$; Set II, $F = 95.57$, $p < 0.001$). The flowering time for the control plants was also significantly different from transgenic plants for Set I ($F = 36.68$, $p < 0.001$) (Figure 5). For set II, the transgenic plants flowered at 21.24 ± 0.65 days post-transplanting, while the control plants had not flowered 30 days post-transplanting.

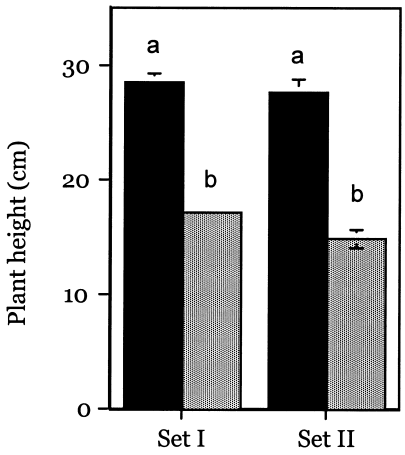

Figure 4. Plant height (cm) of tomato plants at 45 days post-transplanting. Black bars represent the mean plant height for control Beefmaster. Gray bars represent the mean plant height for T2 transgenic Beefmaster B1 line. Vertical lines indicate 1 SE. Columns associated with a different letter are significantly different.

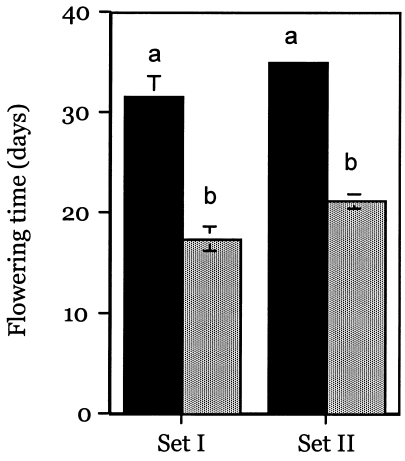

Figure 5. Number of days required for flower bud formation in tomato plants. Black bars represent the average time to flowering for control Beefmaster plants. Gray bars represent the average time to flowering for T2 transgenic Beefmaster B1 line. Vertical lines indicate ± 1 SE. Columns associated with a different letter are significantly different.

The endochitinase and chitobiosidase activities measured at 30 days post planting were positively correlated with the height of the plants measured 35 days post-planting (for endochitinase activity $r^2 = 0.989$, $Y = -0.546 \log (x) + 0.923$ (Figure not shown), for chitobiosidase activity $r^2 = 0.833$, $Y = -3.63 \log (x) + 6.307$). The endochitinase activity and chitobiosidase activity measured at 45 days post-seed planting were positively correlated with the flowering time (for endochitinase activity $r^2 = 1.0$, $Y = -1.889 \log$

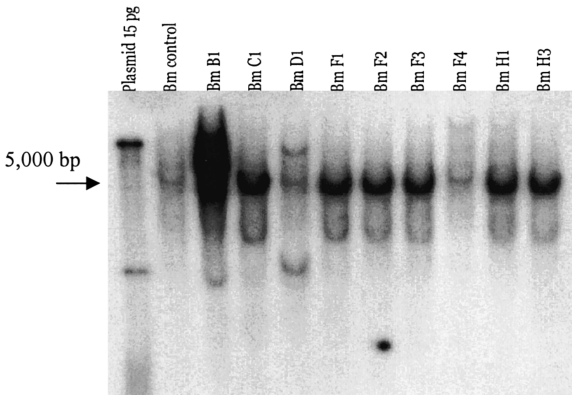

Figure 6. Southern blot of transgenic tomato plants using a T-DNA probe synthesized from the double construct, p*S.a*-endochitinase-chitobiosidase. Lane 1 (plasmid) was loaded with 15 pg of p*S.a*-endochitinase-chitobiosidase digested with *Hin*dIII. All the other lanes were loaded with 15 μg of DNA digested with *Hin*dIII. Lane 2 (Bm control) was loaded with a sample from Beefmaster non-transgenic plant. All the other lines correspond to T1 (transgenic) lines.

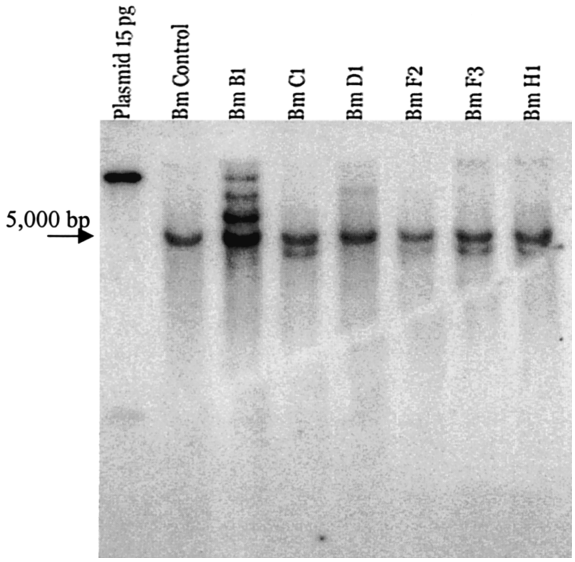

Figure 7. Southern blot of transgenic tomato using a chitobiosidase probe synthesized from chitobiosidase inserted in the plasmid pBluescript II. Lane 1 (plasmid) was loaded with 15 pg of p*S.a*-chitobiosidase (Chitobiosidase inserted in pBin19) digested with *Hin*dIII. All the other lanes were loaded with 15 μg of DNA digested with *Hin*dIII. Lane 2 (Bm control) was loaded with a sample from Beefmaster non-transgenic plant. All the other lines correspond to T1 (transgenic) lines.

(x) + 3.366, for chitobiosidase activity $r^2 = 0.823$, $Y = -6.744 \log (x) + 13.207$).

Relationship between chitinolytic enzyme activity and development of different T1 tomato lines

To eliminate the possibility that the effect on growth and flowering time observed in the T2 transgenic BMB1-1 line was due to a mutational effect caused by the insertion of the transgenes in the plant genome, rather than the expression of the endochitinase and chitobiosidase transgenes, T0 transgenic lines were analyzed. For this, ten seeds from 10 different T0 transgenic lines, transformed with the same construct (p*S.a*-endochitinase-chitobiosidase) as the T2 plants and 12 seeds from control plants were planted.

PCR was performed on DNA leaflet from the first true leaf to identify the presence of *nptII* gene. Putative transgenic plants that were *nptII* negative were discarded. The plants that were *nptII* positive were transplanted individually into soil in pots.

Southern blot analyses were performed on one plant from each line to determine if the complete T-DNA fragment was inserted, and to determine the number of copies per genome. Line B1 showed 5 copies of the T-DNA, lines C1, D1, F1, F2, F3, H1 and H3 showed 2 copies, and line F4 was not transformed (Figure 6). The Southern blot, using the chitobiosidase gene as a probe, showed that the lane that corresponds to the control contained a band around 5 kb. The same band was observed in all the other lines, which indicated that it may correspond to non-specific binding. However, in addition to this band, line B1 showed 3 copies of the genes with molecular weight higher than 5 kb, line D1 showed 1 copy with molecular weight higher than 5 kb and, lines C1, F2, F3 and H1 showed 1 copy with a molecular weight lower than 5 kb (Figure 7). The Southern blot using the endochitinase gene as a probe showed that the lane that corresponds to the control plants contained a band around 5 kb. The same band was observed in all the other lines, which indicated that it may correspond to non-specific binding. However, in addition to this band, line B1 showed 3 copies of the genes with molecular weight higher than 5 kb, lines C1, F1 and H2 showed 1 copy with molecular weight lower than 5 kb, and line D1 showed no copies of the gene. Although, Line D1 did not contain the endochitinase gene, it did contain the chitobiosidase gene (Figure 8).

Plants that were *nptII* positive were evaluated for endochitinase and chitobiosidase activity at 30 days post-seed planting, which was just prior to transplanting to large pots. The plants were allowed to grow for

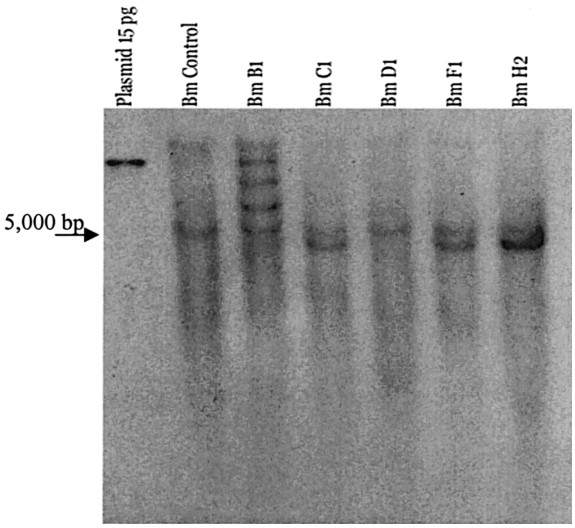

Figure 8. Southern blot of transgenic tomato using the endochitinase probe. Lane 1 (plasmid) was loaded with 15 pg of p*S.a*-endochitinase (endochitinase inserted in pBin19) digested with *Hin*dIII. All the other lanes were loaded with 15 μg of DNA digested with *Hin*dIII. Lane 2 (Bm control) was loaded with a sample from Beefmaster non-transgenic plant. All the other lines correspond to T1 (transgenic) lines.

an additional 15 days (45 days post-seed planting), then the height of the plants was measured. In addition, the endochitinase and chitobiosidase activity was determined at 45 days post-seed planting. At this time the transgenic plants started to flower (Figure 9A and 9B). The statistical comparison (ANOVA) of chitinolytic activity in the T1 lines and control plants at 45 days post-planting (Table 1) indicated that lines B1, F2 and H1 differed from the control for endochitinase and chitobiosidase activity; lines D1, F1 and F3 differed from the control for chitobiosidase activity but not for endochitinase (line D1 did not show the endochitinase gene in the Southern blot); lines C1 and H2 did not differ from the controls for endochitinase or chitobiosidase activity.

When the height of the plants was measured at 45 days post-seed planting (Figure 10), no difference was found between the controls and lines C1 and F1. In all the other lines, the difference was significant with respect to the controls ($F = 6.5$, $p = 0.001$). No difference was found between the number of leaves in the controls and the transgenic lines (data not shown). At 45 days post-seed planting there was a negative correlation between height of the plants and chitinolytic enzymes (for endochitinase $r^2 = 0.785$, $Y = -1.443 \log(x) + 2.423$, $F = 13.76$, $p = 0.010$ (Figure 11), for chitobiosidase activity $r^2 = 0.541$, $Y = -4.3 \log(x) + 8.54$, $F = 5.91$, $p = 0.045$).

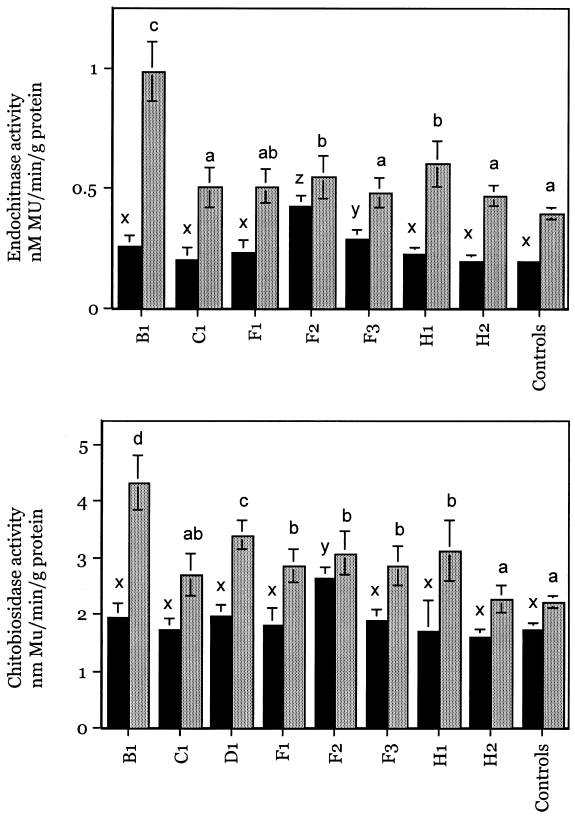

Figure 9. 9A – 9B. Endochitinase and Chitobiosidase activity in the tomato T1 transgenic lines (B1, C1, F1, F2, F3, H1 and H2) and control (non-transgenic) plants. Black bars represent the mean endochitinase or chitobiosidase activity at 30 days post-planting. Gray bars represent the mean endochitinase or chitobiosidase activity at 45 days post-planting. Vertical lines indicate ± 1 SE. Columns associated with the same letter are not significantly different.

When the flowering time in the control plants was compared with the transgenic T1 lines (Figure 12), only lines B1 and D1 differed significantly from the controls ($F = 9.8$, $p = 0.001$). At 45 days post-seed planting there was a negative correlation between days to flower and chitinolytic enzymes [for endochitinase $r^2 = 0.807$, $Y = -2.374 \log(x) + 4.18$, $F = 15.13$, $p = 0.012$; for chitobiosidase $r^2 = 0.832$, $Y = -8.44 \log(x) + 15.8$, $F = 23.62$, $p = 0.003$ (Figure 13)].

Effect of trimming on T1 transgenic tomato line

Between 10 and 20 control plants and T2 BmB1-1 were trimmed. In both trimmed and non-trimmed groups of plants, the control plants differed significantly from the transgenic plants in flowering time

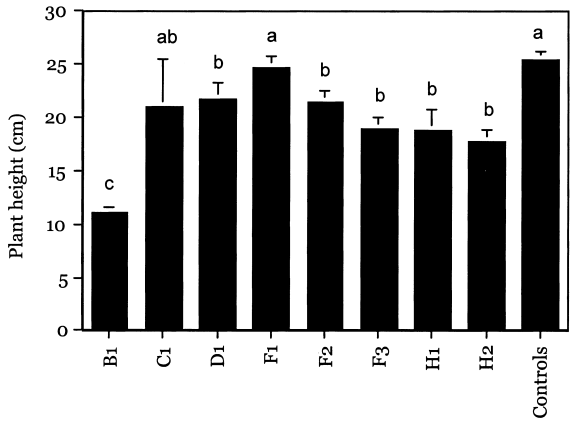

Figure 10. Plant height (cm) of transgenic tomato T1 lines (B1, C1, D1, F1, F2, F3, H1 and H2) and control plants at 45 days post-planting. The bars represent the mean plant height for each T1 transgenic line and control plants. Vertical lines indicate ± 1 SE. Columns associated with the same letter are not significantly different.

Table 1. ANOVA comparison of tomato T1 transgenic lines and control plants with respect to the levels of endochitinase and chitobiosidase activities.

Line	ANOVA Endochitinase activity Control vs. Lines			ANOVA Chitobiosidase activity Controls vs. Lines		
	F	p		F	p	
B1	58.83	< 0.001	c	46.78	< 0.001	d
C1	2.85	0.11	a	3.06	0.111	ab
D1				25.21	< 0.001	c
F1	3.50	0.075	ab	6.41	0.019	b
F2	5.07	0.036	b	8.97	0.007	b
F3	2.53	0.127	a	5.35	0.03	b
H1	6.89	0.018	b	7.24	0.016	b
H2	1.68	0.211	a	0.07	0.789	a
Control			a			a

Lines associated with the same letter are not significantly different.

(Figure 14) (for trimmed plants $F = 82.65$, $p < 0.001$; for non-trimmed plants $F = 92.55$, $p < 0.001$). When comparing the two sets of control plants, the non-trimmed plants were significantly different from the trimmed plants ($F = 9.7$, $p = 0.007$). The control plants that were trimmed flowered in less time (11 days sooner) than the non-trimmed controls. However, the transgenic plants that were trimmed did not differ from the non-trimmed transgenics ($F = 0.04$, $p = 0.838$).

With respect to the number of fruits (Figure 15), the non-trimmed control plants did not produce fruit 60 days post-planting. In the group of plants that was trimmed, the control plants produced significantly fewer fruits than the transgenic plants ($F = 32.4$, $p < 0{,}001$). Fruit production on the transgenic plants that were trimmed did not differ from the non-trimmed transgenics.

Discussion

Our findings suggest that chitinolytic enzymes play an important role in regulating the height of plants, time to flowering, and level of fruit production in tomato plants. Independent of the fact that the plants were transformed with the transgenes from *S. albidoflavus*, in all cases there was an increase of endochitinase and chitobiosidase activity during growth of the plant. This suggests that there is an accumulation and/or increased production of the chitinolytic enzymes during normal development of the plants. This gradual increase in enzyme activity continued until flower buds were produced. Then the levels of enzyme activity remained constant until the fruits matured; at which time the levels decreased significantly (Figures 2 and 3). Transformation of tomato with endochitinase and chitobiosidase genes *S. albidoflavus* significantly decreased the time to production of flower buds by at least 15 days compared with control plants. Analysis of the 10 different T1 lines indicated that the reduced time to flowering and enhanced fruit production was not a result of a mutational effect of the transgenes, since all lines were transformed with the same construct of the bacterial endochitinase and chitobiosidase, and all transformants showed the same response to elevated levels of chitinolytic activity.

The Southern blots showed that most of the T1 lines were transformed with both genes (endochitinase and chitobiosidase). When the Southern blots were probed with the endochitinase or chitobiosidase gene, a band around 5 kb was observed in the control and the transgenic lines. This band may correspond to non-specific binding, or it may be the result of homology with some sequence in the tomato genome. Sequence homology between the chitinolytic genes from *S. albidoflavus* and plant genes has not been reported before, and no consensus sequences were found between those genes and plant genes when an analysis of homology was performed using the data bank "GenBank" (National Center of Biotechnology Information NCBI). In addition to the 5 kb band, the

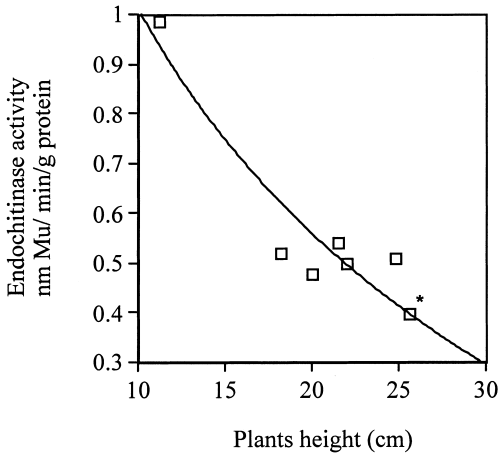

Figure 11. Correlation between endochitinase activity and plant height (cm) at 45 days post-planting for 6 different lines of T1 tomato plants and control plants. Y = – 1.443 log (x) + 2.423 r^2 = 0.785.* Represent the control plants.

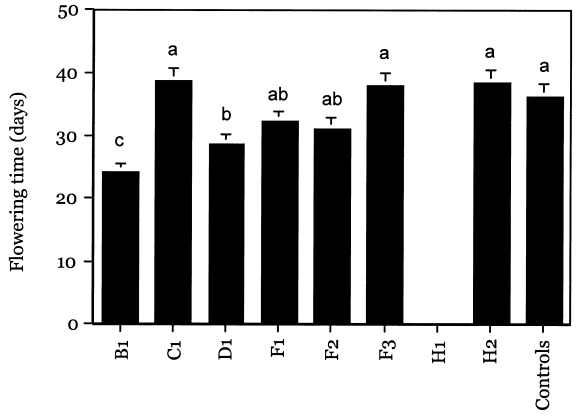

Figure 12. Flowering time (post-transplanting) of transgenic tomato T1 lines (B1, C1, D1, F1, F2, F3, H1 and H2) and control plants. Each bar represent the mean of flowering timefor a single T1 transgenic lines or control plants. Vertical lines indicate ± 1 SE. Columns associated with the same letter are not significantly different.

T1 lines showed other bands that corresponded to the chitinolytic enzymes. When the T1 lines were analyzed for chitinolytic activity, different levels of the enzymes were observed in the different lines, and when the levels of the enzyme activity were evaluated in relation to the height of the plants and the flowering time, a high negative correlation was observed (Figures 11 and 13). This suggest that the effect of the chitinolytic enzymes on the development of the tomato plants is a common characteristic that may be observed in all the tomato lines transformed with the endochitinase and/or chitobiosidase from *S. albidoflavus*.

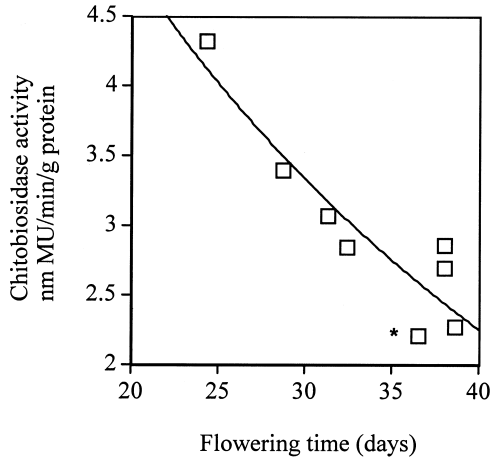

Figure 13. Correlation between chitobiosidase activity and flowering time 45 days post-planting in 7 different tomato T1 lines and control (non-transgenic) plants. Y = – 8.443 log(x) + 15.8 r^2 = 0.832. * Represent the control plants.

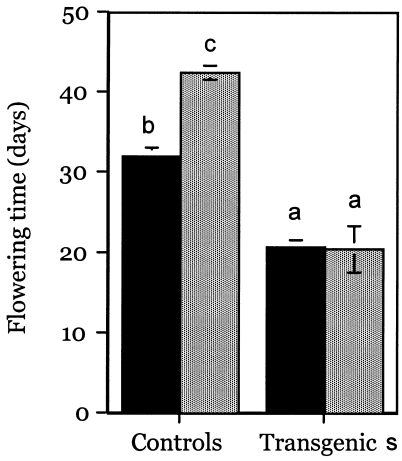

Figure 14. Effect of leaf trimming on flowering time in control plants and T2 transgenic B1-1 line. Black bars represent the mean flowering time in plants that were trimmed. Gray bars represent the mean flowering time in plants that were not trimmed. Vertical lines indicate ± 1 SE. Columns associated with the same letter are not significantly different.

Based on these findings, we propose that the initiation of flower bud formation requires that a threshold level of endochitinases and/or chitobiosidases must be reached in the plant. It has been demonstrated that the morphogenic changes evident in plants during the transition from vegetative to reproductive development are accompanied by the appearance of new gene products (Pierard et al. 1980). We believe that chitinolytic enzymes are part of this group of genes that are induced prior to flowering. In support of our hypothesis we found that transgenic plants contained

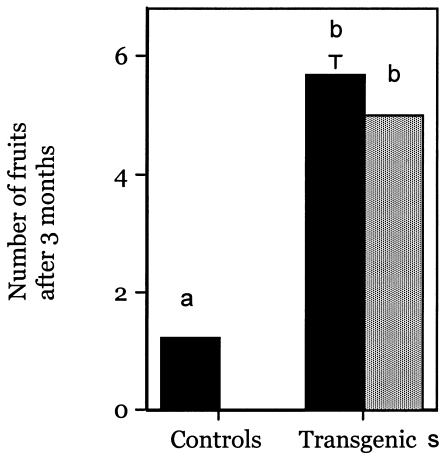

Figure 15. Effect of leaf trimming on the number of fruits produced 60 days post-transplanting of control plants and T2 transgenic B1-1 tomato line plants. Black bars represent the mean number of fruits on plants that were trimmed. The gray bar represents the mean number of fruits on non-trimmed plants (non-trimmed control plants produced no fruits). Vertical lines indicate ± 1 SE. Columns associated with the same letter are significantly different.

higher levels of endochitinase and chitobiosidase than the controls, and the level of enzyme activity was highly negatively correlated with the time to flowering. We suggest that transformation resulted in reducing the time required to reach the threshold level of chitinolytic enzymes, thus flowering was initiated in a shorter period of time (i.e., 15 days) than the control plants.

Our findings in part are supported by previous studies, which indicate that chitinolytic enzymes are involved in the process of flowering. Neale et al. (1990) isolated a group of genes expressed at an early stage of floral bud formation in *in vitro* tobacco (Neale et al. 1990). One family of genes belongs to the family of chitinolytic enzymes and ß-1–3-glucanase and both of them have high homology to chitinolytic enzyme sequences previously reported in tobacco. Some of the genes were also induced by environmental and physiological stress, including pathogen attack. In addition, tobacco mosaic virus and wounding induced the transcription of the genes involved in flowering. The chitinolytic enzymes reported in Neale's study were only induced during the floral bud formation, which means that the total level of chitinolytic enzymes in the plants should increase prior to the floral bud formation, which corresponds with the results observed in our study. Harikrishna et al. (1996) also studied an endochitinase gene isolated from a tomato pistil cDNA library (*Chi2;1*) (Harikrishna et al. 1996). They found that this enzyme was associated with flowering, its expression was temporally regulated, and it was produced predominantly in floral organs. The protein accumulated in the transmission tissue of the style prior to anthesis. In contrast, a low level of the enzyme was present in vegetative tissue. This study provides additional evidence of the increased levels of chitinolytic enzymes prior to flowering.

The experiments involving trimming leaves from the plants also supports our hypothesis that initiation of the process of flower bud formation requires a threshold level of chitinases. Control plants that were trimmed produced flowers in less time than the non-trimmed controls ($F = 9.7$, $p = 0.007$). However, when the flowering time in the trimmed transgenic plants was compared with the flowering time in the non-trimmed transgenics, no difference was found ($F = 0.04$, $p = 0.838$). We suggest that the process of trimming the control plants may cause a wound-induced response that results in hyper-production of chitinolytic enzymes together with other induced factors, such as pathogen, related (PR) proteins (Hammond and Jones 1996). In this way, the threshold of the chitinolytic enzymes, which triggers the formation of flowering buds, is reached in less time in the trimmed plants than the non-trimmed plants. Therefore, the trimmed control plants would be expected to flower in less time than the non-trimmed controls. In the non-trimmed plants, induction did not take place because there was no wounding, and the chitinolytic enzyme threshold is reached at a later (normal) time. In contrast, trimming the transgenic plants did not influence flowering, because, although the trimming process can enhance the production of chitinolytic enzymes, transformation prematurely elevated the levels of chitinolytic enzymes (i.e., the chitinolytic enzyme threshold was reached before trimming) and the trimming process did not have an effect on the transgenic plant. The threshold for flowering was reached independently of the effect of trimming. Not only is the expression of the chitinolytic genes affecting the flowering time in the plants, the process of fruit production is also affected. The non-trimmed control group did not produce fruits after 60 days. The trimmed transgenic plants produced more fruits than the trimmed control plants and, again, no differences were found between trimmed and non-trimmed transgenic plants. Thus, elevating the level of chitinolytic enzyme activity in tomato plants significantly increases fruit yield.

In addition to flower and fruit production, elevated levels of chitinolytic enzyme activity significantly reduce the height of tomato plants during the pre-flowering stage of development. After flowering, the differences in height when comparing control and transgenic lines were not significant (data not shown). However, the effect on height was more strongly correlated with the levels of endochitinase activity than chitobiosidase activity. In a related study we found that extremely high levels of expression of the endochitinase gene may have deleterious effects on plant growth and development. When the endochitinase gene from *S. albidoflavus* was transgenically inserted into apple plants (data not publish), the plants that were regenerated in tissue culture failed to become established in soil, a phenomenon that did not take place in apples transformed with the chitobiosidase gene. This response was also observed in apple plants transformed with another endochitinase gene from *Trichoderma harzianum*. (Dr Jyothi Prakash Bolar, personal communication). In this experiment, the levels of endochitinase activity in the apple plants were more than 10 fold higher than those observed in the tomato plants.

Clearly the levels of chitinolytic enzyme activity are linked to plant development. However, we do not know why chitinolytic enzyme activity increases prior to the flower bud formation, or which substrate is digested by the chitinolytic enzymes. Immunological studies show the presence of the GlcNac residues in the secondary cell walls of plants (Benhamou and Asselin 1989). It is possible that the chitinolytic enzymes play a role in cell wall breakdown by hydrolysing the GlcNac residues. Morphogenic changes that occur during plant development necessitate disruption of existing plant tissues. Such situations may occur during pollen tube growth, the formation of various organs such as lateral or adventitious roots (Varner and Lin 1989), seed germination, leaf senescence and the transition from vegetative to floral meristems (Neale et al. 1990). The direct effect of chitinolytic enzymes on the degradation of the cell wall has been observed in barley seeds where chitinolytic enzymes are induced during inbibition of the seed, resulting in hydrolysis of the wall of the aleurone cells of the endosperm that contains ß-1–3 -glucan, causing the digestionof the aleurone wall, and allowing the emergence of the radicule (Vogeli-Lange et al. 1994). We believe that the formation of the flower bud may involve a similar process. It has been postulated that the action of plant growth regulators may be mediated via oligosaccharides released from plant cell walls by hydrolytic enzymes (Cote and Hahn 1994). If the chitinolytic enzymes can hydrolyze the GlcNac residues and promote the release of those oligosaccharides, they may be involved in signal transduction pathways for developmental events (Hanfrey et al. 1996).

The experiments reported in this study provide further indications that the role of chitinolytic enzymes is not restricted to plant defense. Elevated levels of chitobiosidases and endochitinases in the tomato plants result in reduced plant height, early flowering, and enhanced fruit production. We have not determine whether these effects are exclusively due to the expression of the transgenes of endochitinase and chitobiosidase from *S. albidoflavus* or the additive effect of these 2 enzymes combined with the endogenous chitinolytic enzymes produced by the plants. However, when control plants were trimmed, early flowering was observed compared with the controls that were not trimmed, which indicates that wound induced proteins such as chitinolytic enzymes affect the time to flowering. Further experiments that quantify the total levels of chitinolytic enzymes should be performed in other species of plants, to determine if the pattern observed in tomato could be extrapolated to other species. In addition, transformation of other species of plants with these two genes may help us understand the role of chitinolytic enzymes in the flowering and fruiting processes. In these studies we reported that two genes (endochitinase and chitobiosidase) can significantly reduce developmental time by speeding up the process of flowering and fruiting of tomato, resulting in increased production of fruit. One of the primary goals of all crop breeding programs is to increase the productivity of plants. These two genes should be as candidates for improving plant yield.

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